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Synbiotic effects of the dietary fiber long-chain inulin and probiotic *Lactobacillus acidophilus* W37 can be caused by direct, synergistic stimulation of immune Toll-like receptors and dendritic cells

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List of abbreviations

Acute monocytic leukemia reporter cell line, THP-1

Caco-2 spent medium, Caco-SM

Caco-2 spent medium after exposure to *Salmonella* Typhimurium, Caco-SM-STM

Chemokine C-C motif ligand 2 / monocyte chemoattractant protein 1, CCL-2/MCP-1

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Chemokine C-C motif ligand 3 / macrophage inflammatory protein 1-alpha, CCL3/MIP-1 α

Chemokine C-C motif ligand 5 / regulated on activation normal T cell expressed and secreted, CCL-5/RANTES

Dulbecco's modified Eagle's medium, DMEM

Dendritic cell, DC

heat inactivated Fetal Calf Serum, hiFCS

Human Embryonic Kidney HEK

Intestinal Epithelial Cell, IEC

Lactobacillus acidophilus W37, LaW37

Long chain inulin type fructans, IcITF

Room temperature, RT

Salmonella enterica subspecies *enterica* serovars Typhimurium, STM

Short Chain Fatty Acids, SCFAs

Secreted Embryonic Alkaline Phosphatase, SEAP

Tumor Necrosis Factor alpha, TNF- α

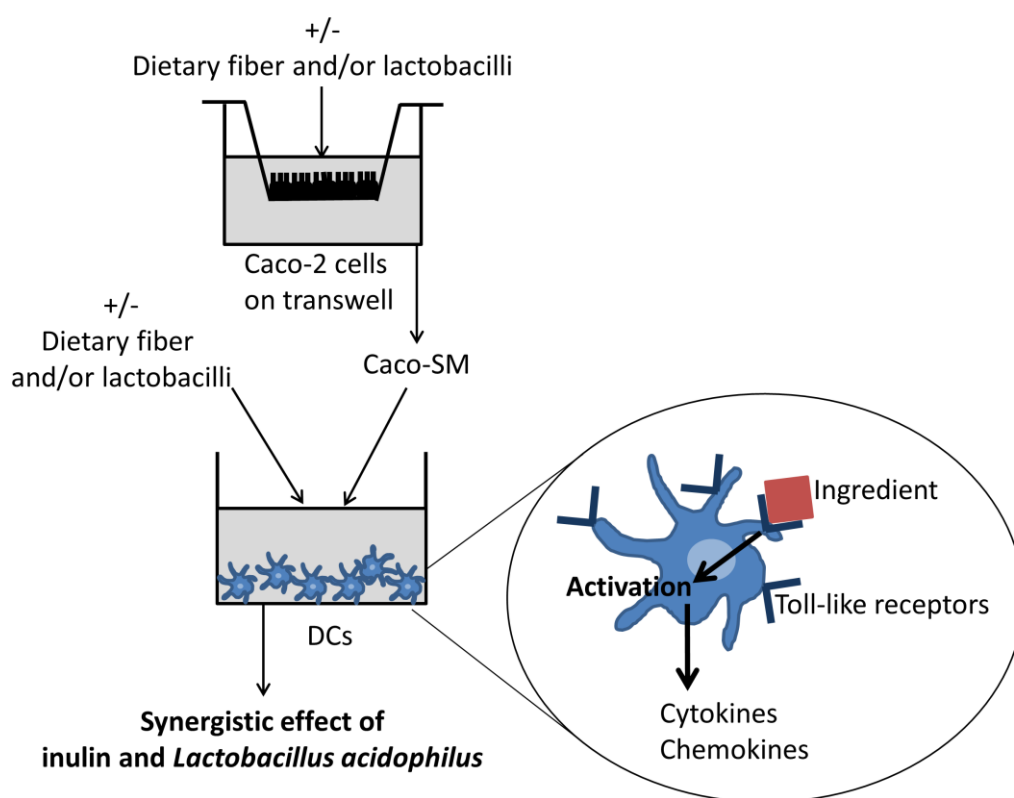
Toll-like receptors, TLRs

Keywords: Caco-2, dendritic cells, inulin type fructans, *Lactobacillus acidophilus* W37, and *Salmonella* Typhimurium.

Abstract

Scope: Synbiotic effects of dietary fibers and lactobacilli are usually explained by synergistic modulation of gut-microbiota. New insight however has demonstrated that both dietary fibers and lactobacilli can directly stimulate immune cells and benefit consumer-immunity. Here we investigated synergistic effects of immune-active long-chain inulin (IcITF) and *L. acidophilus* W37 (LaW37) on dendritic cells (DCs). **Methods and results:** Effects of IcITF and LaW37 alone or combined were studied on Toll-like receptor (TLRs) signaling and cytokine secretion by DCs in the presence and absence of media of intestinal epithelial cell (IEC) exposed to the ingredients. Also, we investigated the effects on DC-responses against *Salmonella* Typhimurium (STM). Synergistic effects were observed on TLR2 and 3. Synergistic effects were not always pro-inflammatory. LaW37 was strongly pro-inflammatory while cytokine-responses were regulatory when combined with IcITF. Exposure of DCs to IECs-medium changed the DCs response which revealed synergistic enhancing effects of IcITF/LaW37 on production of IL-6 and IL-8. DCs response in presence of STM and LaW37 were so strong that IcITF had no additional effect. **Conclusion:** We demonstrated that synbiotic effects of dietary fibers and bacteria are not limited to effects on gut microbiota but can also occur by synergistically directly stimulating IECs and/or immune cells.

Dietary fibers and lactobacilli are daily components of the human diet. Both have been described to directly bind to receptors on immune cells, and have beneficial effects on the consumer's health. It is however unknown whether these dietary fibers and lactobacilli can synergistically directly influence immunity, although synergistic effects of such a combination have already been reported on gut microbiota. Here we tested in a complex culture system the effect of an immune active inulin type fructans, an immune active *Lactobacillus acidophilus* strain, and the combination of both on TLR signaling and on modulation of dendritic cells response. Possible functional effects were tested during stress with the enteropathogen *Salmonella*. The combination synergistically stimulated TLR2 and 3 and modulated dendritic cell responses.



1. Introduction

Both consumption of dietary fibers and of beneficial bacteria such as lactobacilli have been associated with health benefits including lowering symptoms of inflammatory diseases such as inflammatory bowel disease [1-3], and infections with enteropathogens such as *Salmonella* Typhimurium (STM) [4]. Despite those widely accepted beneficial effects of these food ingredients, the mechanisms behind these health effects are still not fully elucidated. It has been shown that consumption of dietary fibers and lactobacilli may lower circulating cytokines [5-9], and that some combinations of fibers and bacteria may have synergistic effects in humans [3, 10, 11]. There is an urgent need for studies to better understand the cellular processes involved in these beneficial immune effects of dietary fibers and/or beneficial bacteria [12], as it may lead to design of more effective strategies to prevent and/or manage inflammatory diseases.

Classically, beneficial effects of food ingredients have been attributed to effects on gut microbiota and stimulation of its beneficial fermentation products such as short-chain fatty acids (SCFAs) [13]. However, evidence is accumulating that immune active food ingredients such as dietary fibers and bacteria can also directly interact with the intestinal immune system [14-17]. For example, we have shown that inulin type fructans (ITF) can stimulate TLRs on intestinal epithelial cells (IECs) [18, 19] and on immune cells resulting in NF- κ B/AP-1 activation and modulation of cytokine release from immune cells [17]. Similar immune effects via pattern recognition receptors have been reported for lactobacilli [14, 16, 20]. As a consequence of these interactions with pattern recognition receptors, these food ingredients may be instrumental for example in management of *Salmonella* Typhimurium (STM) infection as this enteropathogen uses TLRs [21], especially TLR2, 4 and 5, to invade the host [22].

An important intestinal immune cell type involved in sensing of beneficial food ingredients are dendritic cells (DCs). DCs are one of the first immune cell types to come into contact with food compounds in the gut lumen [23, 24]. Their role is to sense and phagocytose antigens and subsequently trigger adequate immune responses in other cells, such as T cells [23]. In the intestine DCs are located on strategic immune signaling locations such as in the lamina propria, mesenteric lymph nodes and between IECs, where they continuously encounter luminal antigens [25]. These luminal antigens may initiate different types of immune responses in DCs. The type of initiated response is determined by the composition of the antigen [26] and (co)stimulation in the gut lumen of the innate immune receptors such as TLRs [17, 27]. In DCs, activation of specific pattern recognition receptors led to different cellular response which can regulate innate and adaptive immunity [23, 24].

The DCs in the intestine experience crosstalk with the IECs which are the first cells to encounter beneficial food components such as dietary fibers and lactobacilli [25]. These IECs release, to be characterized, regulatory factors upon encounter of dietary fibers that attenuate inflammatory responses in DCs [28]. This anti-inflammatory effect is dietary fiber type dependent and may even change T-cell responses in a dietary fiber specific way [17]. Similar effects have also been shown with bacterial food components such as lactobacilli and are strain-dependent [29, 30]. However, possible direct synbiotic effects of dietary fibers and bacteria such as lactobacilli on immune cells have never been studied. Synbiotic effects are up to now mainly attributed to synergistic effects on microbiota [31-33].

Here we studied possible direct synergistic effects of ITF and lactobacilli on DC immune responses. This was done by directly exposing DCs to the food ingredients in the presence and absence of media obtained from cultures of IECs that were exposed to the food ingredients for 20 hours. This design allows us to conclude whether factors released by IECs during exposure to lclTF or a *Lactobacillus* strain modulate DC responses. We chose *L. acidophilus* as study subject as this bacterium is known to modulate immune responses by modulating TLRs [7, 20, 34]. Another reason to choose *L. acidophilus* was that we also studied the effects of ITF and lactobacilli alone and combined in an experimental setup where IECs were infected with STM. *L. acidophilus* is known to compete with STM for adhesion on IECs [35, 36] and to induce direct immune effects which can support DCs response towards the enteropathogen [35, 37]. This allows us to conclude whether ITF and/or lactobacilli can be instrumental in management of STM by directly interacting with intestinal cells.

2. Material and methods

2.1 Ingredients and *Salmonella* Typhimurium culture

L. acidophilus strains W37 (LaW37) (Winclove, Amsterdam, The Netherlands) was produced anaerobically at 37°C, in modified MRS broth. Glycerol stocks were washed with PBS and resuspended in culture medium, brought to 37°C, to reach 10⁷ CFU/mL.

Long-chain inulin type fructans (lclTF) with DP10-60 (Frutafit® Tex!, Sensus, Cosun, Roosendaal, The Netherlands) was solubilized at 10 mg/mL in culture medium at 37°C and further diluted in medium to 5, 1 and 0.5 mg/mL. The ITF was characterized by high-performance anion exchange chromatography coupled with pulsed electrochemical detection, which was performed on an ICS5000 system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a Dionex CarboPac

PA-1 column (2 × 250 mm) in combination with a Carbopac PA-1 guard column (2 × 50 mm) (Supporting Information Figure S1). The solution was 0.2 µm filtered to eliminate possible bacterial contaminations. Endotoxin concentrations in the filtered ICIIF solutions were measured using the Limulus Amoebocyte Lysate (LAL) that was carried out according to the manufacturers protocol from Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific (Pierce Biotechnology, Rockford, USA). Concentrations of endotoxins in ICIIF filtered solutions fell below 0.3×10^{-3} endotoxin units µg⁻¹ (0.002 ng/mL), which is too low to influence the results of the present study.

Salmonella Typhimurium DT12 (STM) was provided by Trouw Nutrition (Boxmeer, The Netherlands). STM was grown in Brain-Heart Infusion (BHI) medium until stationary phase, was washed in PBS, and diluted so that final concentration was 7.5×10^5 CFU / transwell (tw).

2.2 HEK-Blue SEAP reporter cell assays

The human acute monocytic leukemia reporter cell line (THP-1) (InvivoGen, Toulouse, France) expresses endogenously, as previously described [18, 38], the human Toll-like receptors (TLRs) and an inserted construct for Secreted Embryonic Alkaline Phosphatase (SEAP) coupled to the NF-κB and the AP-1 transcription factor responsive promoter. This cell line also carries an extra insert for MD2 and CD14 that boosts TLR signaling. Furthermore, we used 7 different Human Embryonic Kidney (HEK)-BlueTM reporter cell lines expressing one of human TLR2, 3, 4, 5, 7, 8 and 9 (InvivoGen, Toulouse, France). All 7 cell lines also carried the inserted construct SEAP coupled to NF-κB/AP-1 promotor. Upon activation by their respective agonists, NF-κB is transferred to the nucleus, the SEAP gene is expressed and can be measured in the supernatant using QuantiBlue reagent (InvivoGen, Toulouse, France).

THP-1 cells were kept at a concentration of 5×10^5 cells/mL and cultured in RPMI1640 supplemented with 10 % heat-inactivated Fetal Calf Serum (hiFCS), 1.5 g/L NaHCO₃ (Boom B.V. Meppel, The Netherlands), 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate and 50 U/mL penicillin / 50 µg/mL streptomycin, all purchased from Sigma Aldrich Chemie B.V. (Zwijndrecht, The Netherlands) and 100 µg/mL Normocin (InvivoGen). HEK-blue cells were maintained in Dulbecco's modified Eagle's medium (DMEM) culture media (Lonza, Basel, Switzerland) with 10% hiFCS, 2 mM L-glutamine, 4.5 g/L glucose, 50 U/mL penicillin / 50 µg/mL streptomycin, all from Sigma-Aldrich and 100 µg/mL Normocin (InvivoGen). The culture medium was supplemented with selection antibiotics to maintain stable expression of the PRR genes (see Table 1). HEK-Blue cells were grown to approximately 80% confluence and were passaged three times in their respective selection media prior to any experiment, all according to manufacturer's instructions.

2.3 Stimulation of reporter cells with LaW37, IcITF and their combination

The HEK-Blue reporter cell lines were seeded according to Table 1 in 200 μ L/well in a 96 wells flat bottom culture plate and cultured overnight. The following day, medium was refreshed with 100 μ L medium containing IcITF at 0.5, 1, 5 or 10 mg/mL, LaW37 at 10^4 , 10^5 or 10^6 CFU/well, in combination at the following ratio: 10^6 CFU/well / 10 mg/mL; 10^6 CFU/well / 5 mg/mL; 10^5 CFU/well / 10 mg/mL; 10^5 CFU/well / 5 mg/mL, or with the relevant agonist (Table 1), and medium was used as negative control. For the inhibition assays, 10 μ L of the corresponding agonist (Table 1) was added together with LaW37 and/or IcITF. THP-1 cells were incubated with and without addition of 50 μ M Pepinh-MyD88 (InvivoGen).

After 24 hours of incubation at 37°C 5% CO₂, the supernatant of the cells was diluted 1:4 with QuantiBlue solution. After 1-hour incubation, a colorimetric measurement was performed at 650 nm on a Bio-Rad Benchmark Plus microplate spectrophotometer reader (Bio-Rad Laboratories B.V, Veenendaal, the Netherlands) using Bio-Rad Microplate Manager 5.2.1 software. This color change was presented as fold-change of NF- κ B activation. The assay was performed at least five times and each condition was performed in triplicates.

2.4 Caco-2 cell culture and *Salmonella* Typhimurium challenge

ATCC derived Caco-2 cells (HTB-37, 2012) were cultured in DMEM (Gibco-Invitrogen, Bleiswijk, The Netherlands) with 4.5 g/L glucose, 0.58 g/L glutamine, no pyruvate, and supplemented with 10% hiFCS (Hyclone Perbio, Etten-Leur, The Netherlands). Cells were used within passage numbers 30 and 60, and 330,000 were seeded on ThinCert transwells with 33.6 mm² membranes and 3 μ m pores in 24-well suspension culture plates. Cells were grown for 21 days at 5% CO₂ and 37°C. Apical (150 μ L) and basolateral (700 μ L) medium were replaced three times per week and on the day prior to the experiment.

LaW37 and IcITF were prepared within an hour prior to the experiment. Medium was then refreshed with or without the ingredient on the apical side. Each condition was tested in triplicate, and experiments were repeated 6 times, on different days. After 20 hours incubation, the Caco-2 cells were challenged with STM for 45 min after which the cells were washed in PBS apically and basolaterally, and subsequently refreshed with medium containing 100 μ g/mL gentamicin (DMEM^{genta}) on both sides. Basolateral medium of pooled replicates was collected after 4 hours and the supernatant was stored at -80°C for further experiments.

2.5 DCs stimulation

To evaluate the direct immune effects of IcITF and/or LaW37 on intestinal cells we used dendritic cells (DCs) and Caco-2 epithelial cells. DCs were purchased from MatTek (MatTek Corporation, Ashland, MA, USA) and were generated from CD34+ progenitor cells harvested from umbilical cord blood which express HLA-DR, CD83 and CD86, phenotypic maturation markers [17]. The DCs resemble features of DCs found in the gastrointestinal tract, such as presenting antigens to T-cells, and have therefore been previously used in *in vitro* systems mimicking the gastrointestinal track [14-17]. We compared exposure of DCs to the ingredients with and without addition of Caco-2 spent medium (Caco-SM). The design for this series of experiments is described in Figure 1. These experiments were performed 6 times, as previously described [17, 18, 39], on different days.

DCs stimulation experiments were conducted with 2 different settings. First, DCs seeded onto a 96-well plate (40,000 DCs/well) were incubated with 5 mg/mL of IcITF and/or 10^7 CFU/mL of LaW37. After 20 hours, DCs spent medium of triplicates were pooled and stored at -80°C until further analysis (Figure 1A).

Next, we performed the same assay to which we added Caco-SM. This Caco-SM was collected from fully differentiated Caco-2 cells grown on transwells as previously described and exposed to the same ingredient for 20 hours. In this experiment, the DCs are exposed concomitantly to both the ingredients and the Caco-SM collected after Caco-2 cells were themselves exposed to that same ingredient in a 1:10 ratio. After DCs were stimulated for 20 hours, the spent medium of triplicates were pooled and stored at -80°C until further analysis (Figure 1B).

Furthermore, this experiment was repeated after exposure of Caco-2 to STM. In this experiment, Caco-2 cells pre-incubated for 20 hours with ingredients, were challenged with STM for 45 min as described above (2.4). The spent medium collected in the basolateral compartment is then referred to as Caco-SM-STM. Afterwards we proceed as in the previous experiment and stimulated DCs for 20 hours with a simultaneous exposure to Caco-SM-STM and the corresponding ingredient. Spent medium of DCs collected from triplicates were pooled and stored at -80°C until analysis (Figure 1C).

2.6 Luminex analysis of cytokines and chemokines from DCs

The kit Magnetic Luminex® premixed cytokine assay (R&D systems Inc, Minneapolis, USA) was customized to simultaneously measure the following molecules in spent medium from DCs: IL-12/23 p40, IL-1ra, IL-1 β , IL-6, MCP-1/CCL2, CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-10 and TNF- α .

Luminex assays were performed according to the manufacturer's instructions. Briefly, a concentration series of cytokine standards were prepared for the appropriate concentration range. The undiluted microparticle cocktail specific for DCs was added to each well (50 μ L/well), washed, and standards, negative controls, and samples were all incubated O/N at 4°C shaking (duplos, 50 μ L/well). After incubation, the plate was washed three times, a biotin antibody cocktail was added to each well (50 μ L/well) and the plate was further incubated while shaking for 1 hour at room temperature (RT). The plate was washed 3 times and streptavidin-phycoerythrin was added to each well (50 μ L/well). After 30 minutes incubation shaking at RT, the plate was washed three times and the microparticles in were resuspended in 100 μ L of wash buffer. Fluorescence was then measured within 90 minutes using a Luminex® analyzer MAGPIX and xPONENT 4.2 for MAGPIX software (Luminex Corporation, 's-Hertogenbosch, The Netherlands).

2.7 ELISA analysis of IL-8 produced by DCs

IL-8 was measured in spent medium from DCs using a human CXCL8/IL-8 DuoSet ELISA ELISA kit (R&D systems), performed according to manufacturer's instruction. In short, 96-well plates (R&D systems) were coated with capture antibodies at a concentration of 4 μ g/mL O/N at RT after which the plates were washed with a filtered block buffer containing 1% BSA (Sigma Aldrich). Samples were diluted 1:4 and incubated for 2 hours after which the detection antibody was added at a concentration of 20 ng/mL and incubated for 2 hours. Reaction with 40-fold diluted Streptavidin-Horse Radish Peroxidase occurred afterwards for 20 min and was followed by 20 min reaction with 3,3',5,5'-Tetramethylbenzidine (Sigma Aldrich) that was stopped with Carboxylic acid (BioLegend Inc., San Diego, CA, USA). Plates were thoroughly washed 3 three times with wash buffer containing 0.05% TWEEN. Optical density was immediately estimated at 540 nm with subtraction of 450 nm background using Bio-Rad Benchmark Plus microplate spectrophotometer reader. Data were processed using GraphPad Prism version 7.0a (GraphPad Software, Inc., La Jolla, USA).

2.8 Statistical analysis

THP-1 and HEK-blue cells TLRs data were normalized compared to medium control so that medium equals 1, and activation value can be expressed as fold-change induction of NF- κ B/AP-1 compared to medium. The data were not normally distributed as confirmed by the Kolmogorov-Smirnov test. Statistical differences were analyzed using Kruskal-Wallis followed by Dunn's *post hoc* test. Cytokines data were normally distributed as confirmed by the Kolmogorov-Smirnov test and ANOVA was applied followed by LSD test. *p*-values <0.05 were considered to be statistically significant and *p*<0.1 was a trend. Cytokines data are expressed as average (pg/mL) \pm SEM. All data were analyzed with GraphPad Prism.

3. Results

The aim of this study was to determine whether direct immune effects of dietary fibers such as lclTF and a lactic acid bacterium like LaW37 are synergistic and modulates mucosal immunity during infection with an enteropathogen by directly interacting with immune receptors. We first established possible activation of crucial pathogens recognition receptors, the TLRs. Next, we investigated whether these effects were associated with immune cell signaling in DCs, and whether this response was modulated by factors released by IECs after Caco-2 were challenged or not with STM. At last, we evaluated the effects of the ingredients on DCs with and without STM challenge.

3.1 Immune receptors are specifically reacting to LaW37, lclTF and their combination has synergistic effects.

As both lclTF and lactobacilli have been shown to signal on immune cells via TLRs [18, 20], we first investigated possible synergistic effects on reporter cell-lines expressing TLR2, 3, 4, 5, 7, 8 and/or 9. Activation by lclTF was observed in TLR2, 3 and 5 expressing cell lines. Therefore, only these results are shown in Figure 2. lclTF had a strong dose-dependent activating effect on TLR2 (Figure 2A) up to 3.5-fold (*p*<0.0001) at 5 mg/mL. Activation of TLR3 was reaching 1.5-fold (*p*=0.001) at 5 mg/mL (Figure 2B). lclTF induced activation of TLR5 was dose-dependent (Figure 2C) with a 5.6-fold increase (*p*<0.0001) at 5 mg/mL.

LaW37 statistically significantly activated TLR2 in a dose-dependent way inducing a 1.4-fold increase at 10^6 CFU/mL (*p*=0.003) and a 8.2-fold increase (*p*<0.0001) at 10^7 CFU/mL (Figure 2A). A trend towards increase of TLR3 was observed at 10^6 CFU/mL (1.2-fold; *p*=0.07) and a statistically

significant increase reached 5.5-fold at 10^7 CFU/mL ($p=0.0002$) (Figure 2B). The combination lclTF/LaW37 had synergistic effects in TLRs.

The combination activated TLR2 in a dose-dependent manner with a 5.7-fold increase at 5 mg/mL / LaW37 10^6 CFU/mL ($p=0.003$) and 11.4-fold increase at lclTF 10 mg/mL / LaW37 10^6 CFU/mL ($p<0.0001$) (Figure 2A). It also activated TLR3 in a synergistic way. lclTF 10 mg/mL / LaW37 10^6 CFU/mL led to 6.1-fold increase ($p<0.0001$) (Figure 2B). lclTF/LaW37 statistically significantly activated TLR5 in a dose-dependent manner (Figure 2C), this however did not differ from what was observed for lclTF alone.

3.2 LaW37 stimulates a pro-inflammatory phenotype in DCs while lclTF/ LaW37 attenuates the response in DCs

After confirming immune signaling via TLRs and synergistic effects of lclTF and LaW37, we studied the effect of lclTF and LaW37 on DCs to determine the final effects on immune signaling (Figure 1A). We investigated the effects of lclTF, LaW37 and their combination on human IL-12/23 p40, IL-1ra, IL-1 β , IL-6, IL-8, MCP-1/CCL2, CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-10 and TNF- α production (Table 2).

The production of IL-1 β , CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-12.23p40 and IL-10 was below detection levels at all occasions. There was no statistically significant effect of lclTF on DCs. This was different for LaW37. LaW37 statistically significantly increased CCL-2/MCP-1 ($p=0.022$), IL-6 ($p=0.001$) and TNF- α ($p=0.001$) compared to unstimulated DCs (Table 2). Finally, the combination lclTF/LaW37 statistically significantly increased TNF- α ($p=0.026$) and tended to increase CCL-2/MCP-1 ($p=0.084$) (Table 2). In general, the effects of the combination lclTF/LaW37 were milder than those observed for LaW37 as pro-inflammatory IL-6 ($p=0.019$) and TNF- α ($p=0.027$) were statistically significantly higher enhanced by LaW37 than by lclTF/LaW37.

3.3 DC responses to lclTF and/or LaW37 are modulated by intestinal epithelial cells

As it is well known that IECs derived factors are essential for modulating responses of DCs when exposed to dietary fibers [17, 25], we repeated the experiment to investigate whether IECs spent medium (Caco-SM), collected after 20 hours exposure to lclTF, LaW37 or lclTF/LaW37, can modulate the DCs response (Figure 1B). This was done by incubating DCs in a 1:10 ratio of Caco-SM for 20 hours. We abandoned cocultures of IECs and DCs and preferred to include Caco-SM instead with DCs as this set up allows us to exclusively measure the DCs response. We investigated the effects of lclTF, LaW37 or lclTF/LaW37 on human IL-12/23 p40, IL-1ra, IL-1 β , IL-6, MCP-1/CCL2, CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-10 and TNF- α production in spent medium from DCs as presented in Table 3.

First, we assessed the effect on DCs of the Caco-SM itself and compared cytokine levels to that of unstimulated DCs. As shown in Table 3, there was no statistically significant difference for the tested cytokines and chemokines.

Next, we exposed DCs to both the ingredients and Caco-SM after Caco-2 cells were themselves exposed for 20 hours to the same ingredients. The production of IL-1 β , CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-12.23p40 and IL-10 was below detection levels at all occasions. There was no statistically significant difference for lclTF. This was different for LaW37. LaW37 statistically significantly increased the pro-inflammatory chemokine IL-8 ($p<0.0001$) and tended to increase TNF- α ($p=0.064$) and IL-1ra ($p=0.105$) compared to DCs exposed to unstimulated Caco-SM (Table 3). Finally, the combination lclTF/LaW37 statistically significantly increased IL-8 ($p<0.0001$), TNF- α ($p=0.0035$) and IL-6 ($p=0.023$) (Table 3). The combination lclTF/LaW37 had stronger effects on pro-inflammatory cytokines than LaW37 alone and increased the production of the pleiotropic IL-6 but not of the anti-inflammatory IL-1ra, which was the opposite for LaW37 alone. Moreover, synergistic effects by lclTF/LaW37 were observed on IL-8 production as IL-8 tended to be higher enhanced by lclTF/LaW37 than by LaW37 ($p=0.055$) and lclTF ($p<0.0001$) alone.

3.4 STM challenge of Caco-2 cells lowers TNF- α responses in DCs response

Next, we applied a STM challenge to Caco-2 cells to study effect of lclTF, LaW37 or lclTF/LaW37 on STM induced inflammatory responses in IECs. STM is an enteropathogen capable of escaping the immune recognition, invading epithelial cells and being internalized by DCs [40, 41]. We repeated the above experiment on DCs but added a 45 min STM challenge of Caco-2 cells after they were pre-incubated for 20 hours with lclTF, LaW37 or lclTF/LaW37 (Figure 1C). The medium collected from the basolateral side of the Caco-2 culture exposed to both ingredients and STM is further referred to as Caco-SM-STM.

As control, we first analyzed the cytokine levels produced by DCs after exposure to Caco-SM-STM in the absence of ingredients by measuring production of human IL-12/23p40, IL-1ra, IL-1 β , IL-6, MCP-1/CCL2, CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-10 and TNF- α after 20 hours incubation. This was compared to responses of DCs that were exposed to Caco-SM without *Salmonella* Typhimurium exposure. When DCs were exposed to Caco-SM-STM, the production of IL-1 β , CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-12/23p40 and IL-10 was below detection levels at all occasions. As presented in Table 4, challenge of Caco-2 with STM did not induce any statistically significant change in CCL-2/MCP-1, IL-1ra, IL-6 or IL-8. However, TNF- α was reduced from 10 pg/mL in medium control to over 4 pg/mL ($p<0.0001$) in DCs exposed to Caco-SM-STM compared to DCs exposed to Caco-SM control (Table 4).

Next, we analyzed the effects on DCs of lclTF, LaW37 and lclTF/LaW37 on STM challenged IECs. We therefore repeated this experiment with pre-incubation of Caco-2 cells to lclTF, LaW37 or lclTF/LaW37 for 20 hours prior to the 45 min STM challenge. This Caco-SM-STM was then exposed to DCs cultured separately in a 1:10 ratio. DCs were concomitantly exposed to Caco-SM-STM and freshly prepared lclTF, LaW37 or lclTF/LaW37. DCs response was analyzed by measuring production of human IL-12/23 p40, IL-1ra, IL-1 β , IL-6, MCP-1/CCL2, CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-10 and TNF- α after 20 hours incubation. Production of IL-1 β , CCL3/MIP-1 α , CCL-5/RANTES, IFN- γ , IL-12/23p40 and IL-10 was below detection level at all occasions. As presented in Table 4, lclTF had no statistically significant effect on any of the detectable cytokines and chemokines. This was different for LaW37. LaW37 increased all detectable cytokines and this was statistically significant for CCL-2/MCP-1 ($p=0.004$), IL-6 ($p=0.007$), IL-8 ($p<0.0001$) and TNF- α ($p=0.0001$) but not for IL-1ra. The same was observed for the combination lclTF/LaW37.

4. Discussion

Our study shows for the first time, to the best of our knowledge, that dietary fibers and bacterial supplementation can synergistically influence immunity by directly interacting with immune cell receptors, and eventually modulate DC responses. This might be another mechanism for synbiotic effects than the microbiota driven and indirect modulation of immunity so far observed in other studies [31-33]. Also, our study shows that DCs directly exposed to LaW37 or lclTF/LaW37 react differently than DCs that are also exposed to IECs media. In the first case, direct effects of LaW37 were dampened by addition of lclTF, while enhancing effects on IL6 and IL8 were observed in presence of IECs.

The selected dietary fiber lclTF showed direct stimulatory effects on TLR2, 3 and 5. Effects on TLR2 and 5 were dose-dependent as reported before [18]. Despite this observation, direct stimulation of DCs did not lead to any change in cytokine and chemokine production. This can be explained by differential and simultaneous stimulation of pattern recognition receptors leading to activation of several downstream pathways, bringing regulatory and pro-inflammatory responses into balance, as reported before [17, 38]. Another factor could be due to varying expression of TLRs as different DC population may have different phenotypic reactions to TLR activation [42]. Despite absence of immune stimulation of DCs by lclTF we decided to test effects of lclTF in the context of a STM infection of IECs as lclTF stimulates, as shown in our study, TLR5. The main ligand for TLR5 is flagellin, a pathogenic molecule expressed by STM. Unfortunately, during STM infection lclTF did not enhance DCs responses.

Stimulatory effects of the *Lactobacillus* strain LaW37 were observed on TLR2 and 3. This corroborates observation of others [7]. TLR2 activation should be explained by production of lactic acid and presence of lipoteichoic acid on LaW37, which are both specific ligands of TLR2 [20, 34]. Although change of pH and production of lactic acid were not quantified, acidification of the medium was not observed during stimulation of the cells suggesting low metabolic activity of LaW37. Notably, however, the potential of LaW37 to activate TLRs is specific as not all lactobacilli were reported to activate TLRs, *e.g.* *L. paracasei* [14]. The effects of LaW37 on direct immune stimulation were confirmed by exposing DCs to LaW37 which resulted in enhanced production of the chemokine CCL-2/MCP-1 and the pleiotropic IL-6. It also tended to increase the pro-inflammatory cytokine TNF- α which is a more general feature of lactic acid producing bacteria [43]. Moreover, in this same study, effects of *L. acidophilus* were shown to be specific and particularly efficient at increasing expression of HLA-DR, CD40 and CD86 DCs surface maturation markers [43] which are also present on the DCs used in our study and could explain the pro-inflammatory effects.

IEC media modulated responses of DCs exposed to LaW37. Production of the pro-inflammatory cytokines and chemokines IL-8 and TNF- α was increased in the presence of IEC media after Caco-2 cells were exposed to LaW37. Moreover, it implies that bacteria such as *L. acidophilus* may have other effects on IECs than dietary fibers as in a previous study we showed that dietary fibers exclusively attenuate immune responses in DCs [17]. Our observation is in line, however, with studies of others demonstrating the capability of lactobacilli to induce pro-inflammatory responses *in vivo* [6] and in DCs [44]. Notably, induction of IL-8 in DCs was not observed unless Caco-SM was present suggesting that induction of IL-8 production by DCs is likely to be a consequence of IEC derived factors induced by lactobacilli.

The combination of LcITF and LaW37 had synergistic effects on TLR2 and 3 activation which suggest that the mechanisms behind synbiotic effects might also include synergistic effects on immune receptors such as TLRs. As effects on inflammatory responses of combined stimulation of pattern recognition receptors is difficult to predict [15], we also performed studies of synbiotic effect on immune response of DCs. Direct exposure to LcITF/LaW37 led to increased CCL-2/MCP-1 and TNF- α . This increase was statistically significantly lower than for LaW37 alone. This indicates that LcITF/LaW37 together give different responses in DCs and thus have synergistic effects which can be attenuation of inflammatory responses and induction of more regulatory responses. Interestingly, this has already been shown on IECs, also with a combination of LcITF and *L. acidophilus* [45].

Synergistic effects of LcITF/LaW37 were also observed when Caco-SM was added to DCs. LcITF/LaW37 induced production of IL-8 and TNF- α . IL-8 increase was higher for the combination than for LaW37 alone. Moreover, LcITF/LaW37 also induced IL-6 production, which was unique to the combination. To study whether the observed synergistic effects can have any functional meaning, DCs were, next, exposed to IECs infected with STM. After STM challenge, LcITF/LaW37 increased CCL-

2/MCP-1, TNF- α , IL-8 and IL-6 production by DCs. There was no difference compared to LaW37. This is different than the results of Huang et al. where gene expression of IL-8 and TNF- α in IECs during STM challenge was decreased by *L. acidophilus* alone or combined with lclTF [45]. Our experiments solely focused on DCs responses which were not directly exposed to STM, as we mimicked the *in vivo* situation where DCs are protected by the IECs. Therefore, during STM challenge, DCs in our experiments were not under a pro-inflammatory setting unlike the IECs used in Huang et al. study which might explain such differences. Another difference is that the combination used by Huang et al. had stronger effects than *L. acidophilus* alone [45] which suggests that synbiotics can have a synergistic effect during STM infection, although no synergistic effect could be shown in our study. Moreover, in our study, LaW37 already strongly enhanced CCL-2/MCP-1, TNF- α , IL-8 and IL-6 that it is likely that no significant effect of the modest cytokine enhancing dietary fiber lclTF can be measured.

In conclusion, we demonstrated that synbiotic effects of the dietary fiber lclTF and lactic bacteria LaW37 occurred by synergistically activating immune receptors. In general, LaW37 displayed pro-inflammatory effects on DCs no matter the conditions which is likely due to its capacity to stimulate TLR2 and 3. The combination lclTF/LaW37 was found to synergistically activate specific TLRs and had unique effects on DCs cytokine profiles. However, during STM challenge, no added effects of the combination were observed which should probably be explained by the strong effects of LaW37 on DCs cytokine response in the presence of STM infection. This demonstrates for the first time, to the best of our knowledge, that synbiotic effects of dietary fibers and bacteria are not limited to effects on gut microbiota but can also occur by synergistically directly stimulating IECs and/or immune cells. The final outcome of such a synergistic effect depends on the strength by which the individual ingredient modulates immune responses.

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Figures legends

Figure 1. Experimental design for DCs stimulation with lclTF, LaW37 and lclTF/LaW37, in absence (A) and presence (B) of Caco-SM, with (C) and without STM challenge. Effects of long-chain inulin type fructans (lclTF), *Lactobacillus acidophilus* W37 (LaW37) and lclTF/LaW37 on dendritic cells (DCs) directly exposed to these ingredients for 20 hours (A) was compared to a cross-talk situation where DCs are also exposed to the spent medium collected from Caco-2 cells (Caco-SM). For this purpose, Caco-2 cells were cultured on transwells for 21 days in a separate plate and were incubated for 20 hours with lclTF, LaW37 or lclTF/LaW37. The Caco-SM collected was then transferred to the DCs that were separately cultured (B). In this setting DCs are exposed concomitantly to this Caco-SM and the corresponding, freshly prepared, ingredient. At last, Caco-2 cells were challenged for 45 min to *Salmonella* Typhimurium (STM) and the same experiment was repeated (C).

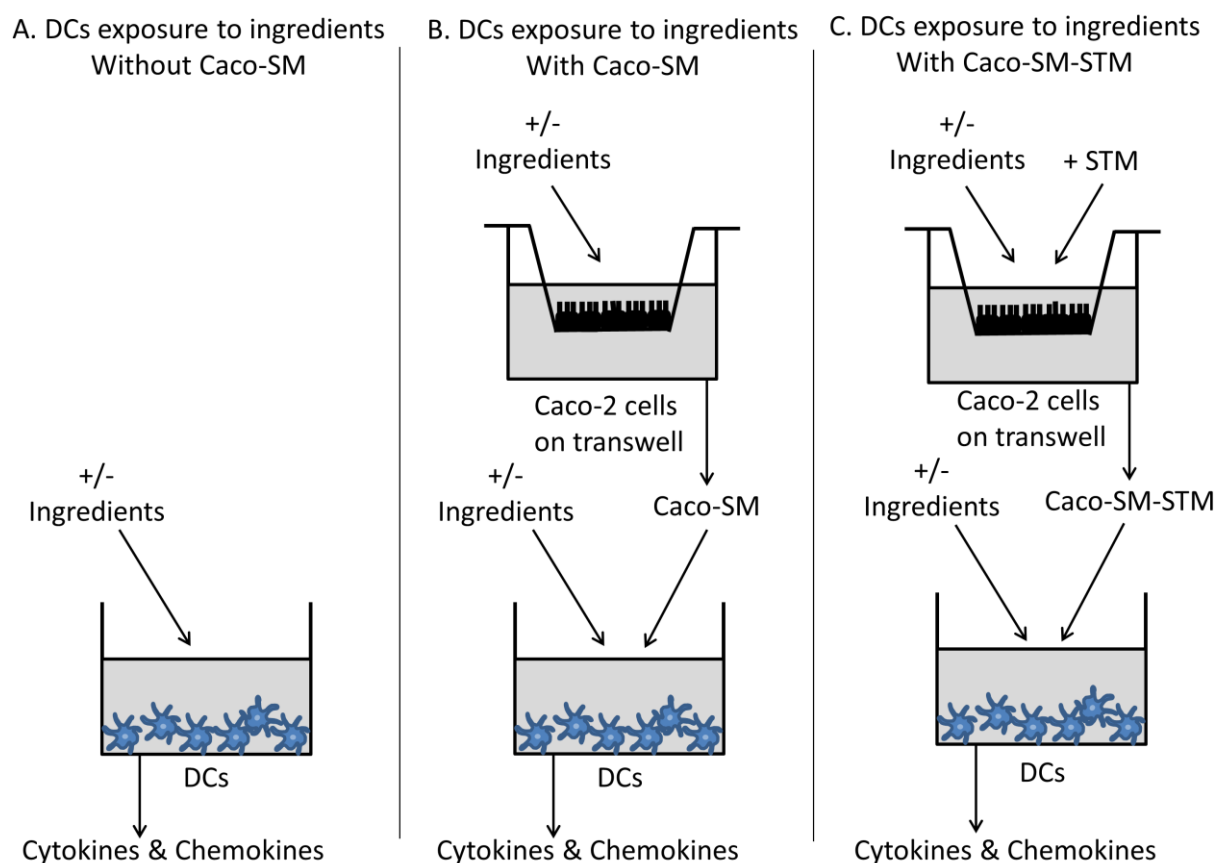
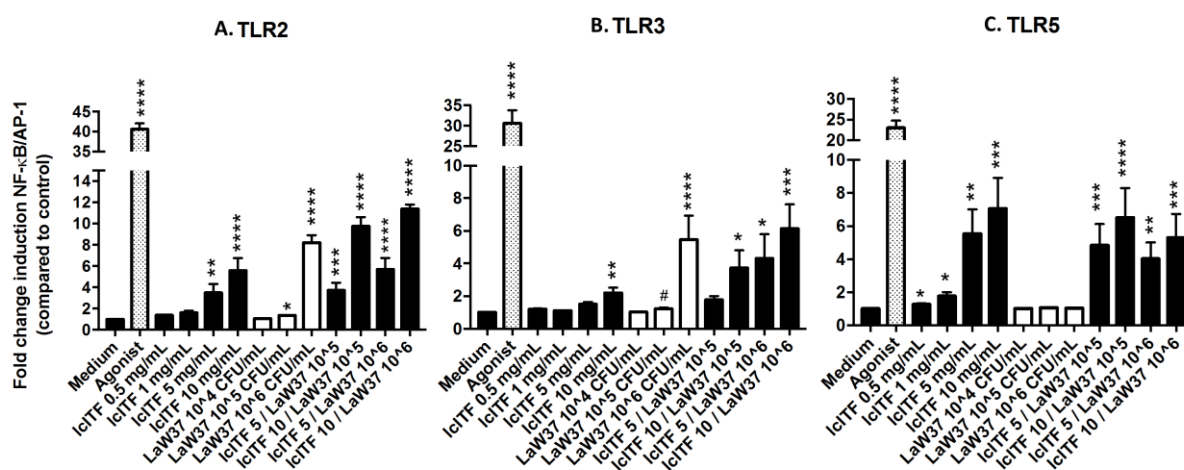


Figure 2. NF- κ B/AP-1 activation in HEK-Blue reporter cell lines expressing TLR2 (A), 3 (B) or 5 (C) by lclTF, LaW37 and lclTF/LaW37. Agonists used were respectively Heat Killed *Lysteria Monocytogenes*, Poly (I:C) Low Molecular Weight and *Salmonella* Typhimurium flagellin. Long-chain inulin type fructans (lclTF) was tested at 0.5, 1, 5 and 10 mg/mL, and *Lactobacillus acidophilus* W37 (LaW37) was tested at 10^4 , 10^5 and 10^6 CFU/mL. n=5 performed in triplicates. Statistical significances compared to medium were assessed by Kruskal-Wallis with Dunn's test and * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and a trend is # $p<0.1$.



Author contributions

AFPL and PdV conceived and designed the experiments. AFPL performed the experiments. AFPL analyzed the data. AFPL and PdV wrote the paper.

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Conflict of interest statement

The authors have declared no conflict of interest

Table 1: Culturing specificities and agonists used in the HEK-Blue reporter assays.

HEK-Blue cell line overexpressing	Selection antibiotics	Cell density (cells/mL)	Positive control (agonist)
TLR2	HEK-Blue (1 μ L/mL)	2.8×10^5	Heat-killed <i>Listeria Monocytogenes</i> (HKLM, 10^8 cells/mL)
TLR3	Blasticidin (30 μ g/mL) Zeocin (100 μ g/mL)	2.8×10^5	Poly (I:C) Low Molecular Weight (LMW, 1 μ g/mL)
TLR4	HEK-Blue (1 μ L/mL)	1.4×10^5	<i>E. coli</i> K12 Lipopolysaccharide-HEK Ultrapure (LPS, 0.1 μ g/mL)
TLR5	Blasticidin (30 μ g/mL) Zeocin (100 μ g/mL)	1.4×10^5	Recombinant flagellin isolated from <i>S. Typhimurium</i> (RecFLA-ST, 0.1 μ g/mL)
TLR7	Blasticidin (10 μ g/mL) Zeocin (100 μ g/mL)	2.2×10^5	9-benzyl-8 hydroxyadenine derivative (CL264, 5 μ g/mL)
TLR8	Blasticidin (30 μ g/mL) Zeocin (100 μ g/mL)	2.2×10^5	20-mer phosphorothioate single stranded RNA is complexed with the transfection reagent LyoVec (ssRNA40/LyoVec, 2 μ g/mL)
TLR9	Blasticidin (10 μ g/mL)	4.5×10^5	Type B CpG oligonucleotide

	Zeocin (100 µg/mL)		(ODN2006, 10 µM µM)
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Table 2. Effect of direct exposure of lclTF, LaW37 and lclTF/LaW37 on DC cytokine responses.

Effects of 5 mg/mL long-chain inulin type fructans (lclTF), 10^7 CFU/mL *Lactobacillus acidophilus* W37 (LaW37) and lclTF/LaW37 on IL-1ra, IL-6, IL-8, MCP-1/CCL2, and TNF- α production was measure by Luminex in medium of DCs directly exposed to ingredients for 20 hours. Data are averages with SEM values of 6 repetitions, with triplicates. Statistical significances of differences compared to unstimulated DCs were tested in GraphPad Prism ANOVA with LSD test and *** $p < 0.001$, * $p < 0.05$ and # $p < 0.1$.

DCs exposed to	Unstimulated DCs		lclTF		LaW37		lclTF/LaW37	
pg/mL	average	SEM	average	SEM	average	SEM	average	SEM
CCL-2/MCP-1	64,1	8,6	43,9	9,3	154,9*	33,5	129 [#]	32,5
IL-1ra	1587	107	1228	147,3	2244	283,2	3270	1074
IL-6	2,1	0,6	2	0,5	22,3***	6,8	9,7	2,9
TNF-a	3,5	0,7	3,3	0,8	27,4***	5,8	16,1*	3
IL-8	565,8	167,7	912,8	247,7	674,6	157,9	683,1	113,2

Table 3. Effect of direct exposure of lclTF, LaW37 and lclTF/LaW37 on DC cytokine responses in the presence of IEC-media. The effects of 5 mg/mL long-chain inulin type fructans (lclTF), 10^7 CFU/mL *Lactobacillus acidophilus* W37 (LaW37) and lclTF/LaW37 on IL-1ra, IL-6, IL-8, MCP-1/CCL2, and TNF- α production by DCs after 20 hours exposure to the ingredients combined with Caco-2 intestinal epithelial cells (IECs) medium (Caco-SM) was measured by Luminex. Caco-2 cells were incubated 20 hours, on beforehand, with lclTF, LaW37 or lclTF/LaW37. Data are averages with SEM values of 6 repetitions, with triplicates. Statistical significance of differences compared to DCs exposed to unstimulated Caco-SM were tested in GraphPad Prism ANOVA with LSD test and **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$ and # $p < 0.1$.

DCs exposed to	Caco-SM		lclTF + Caco-SM		LaW37 + Caco-SM		lclTF/LaW37 + Caco-SM	
pg/mL	average	SEM	average	SEM	average	SEM	average	SEM
CCL-2/MCP-1	84,4	12,7	68	9	124,4	16,4	119,2	14,2
IL-1ra	1575	124	1288	118,3	1982 [#]	258,2	1885	180,8
IL-6	5,8	1,8	6,4	1,9	13,7	5,4	21,4*	5,5
TNF- α	10,5	2,3	8	2	19,6 [#]	2,9	23,6**	4,2
IL-8	556,3	67,9	475,4	51,0	1117****	85	1367****	106,9

Table 4. Effect of direct exposure of IcITF, LaW37 and IcITF/LaW37 on DC cytokine responses in the presence of IEC-media exposed to *Salmonella Typhimurium* DT12 (STM) in combination with the ingredients. The effects of 5 mg/mL long-chain inulin type fructans (IcITF), 10^7 CFU/mL *Lactobacillus acidophilus* W37 (LaW37) and IcITF/LaW37 on IL-1ra, IL-6, IL-8, MCP-1/CCL2, and TNF- α production by DCs after 20 hours exposure to the ingredients combined with Caco-2 intestinal epithelial cells (IECs) medium (Caco-SM-STM) was measured by Luminex. Caco-2 cells were incubated 20 hours, on beforehand, with IcITF, LaW37 or IcITF/LaW37 and challenged with STM for 45 min. Data are averages with SEM values of 6 repetitions, with triplicates. Statistical significance of differences compared to DCs exposed to Caco-SM-STM, without ingredients, were tested in GraphPad Prism ANOVA with LSD test and **** $p < 0.0001$, *** $p < 0.001$ ** $p < 0.01$ and * $p < 0.05$.

DCs exposed to	Caco-SM-STM		IcITF + Caco-SM-STM		LaW37 + Caco-SM-STM		IcITF/LaW37 + Caco-SM-STM	
pg/mL	average	SEM	average	SEM	average	SEM	average	SEM
CCL-2/MCP-1	70,6	17,4	93,8	13,8	137,3**	17,9	124,6*	14,9
IL-1ra	1502	195	1488	81,4	1825	182,9	1794	175,2
IL-6	1,8	0,4	6,5	3	22,7**	6,6	27,8***	7,9
TNF- α	7,3	2,4	6,7	1,2	27***	4,3	26,6***	3,9
IL-8	517,7	87,1	577,8	70,7	1258*** *	140,7	1422****	116,1